Communal metabolism by *Methylococcaceae* and *Methylophilaceae* is driving rapid aerobic methane oxidation in sediments of a shallow seep near Elba, Italy

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Originality-Significance Statement

Methane is a potent greenhouse gas contributing substantially to global warming, and emissions from marine seeps contribute up to 10% of methane in the atmosphere. Methanotrophic microorganisms can use methane as carbon and energy source, and thus significantly mitigate global methane emissions from seep areas, acting as an important 'benthic filter'. This study reports on the efficiency and function of the 'benthic filter' at a shallow methane seep, by quantifying the rates of methane oxidation, identifying the microbial key players involved in this process and assessing their function. Compared to the well-studied deep-sea seeps, shallow seeps represent distinct hydrogeochemical settings, where the risk of emitted methane reaching the atmosphere is much higher. The findings we present are highly relevant to evaluate the impact of shallow seeps on global atmospheric methane budgets.

Abstract

Release of abiotic methane from marine seeps into the atmosphere is a major source of this potent greenhouse gas. Methanotrophic microorganisms in methane seeps use methane as carbon and energy source, thus significantly mitigating global methane emissions. Here we investigated microbial methane oxidation at the sediment-water interface of a shallow marine methane seep. Metagenomics and metaproteomics, combined with ^{13}C-methane stable isotope probing, demonstrated that various members of the gammaproteobacterial family *Methylococcaceae* were the key players for methane oxidation, catalyzing the first reaction step to methanol. We observed a transfer of carbon to methanol-oxidizing methylotrophs of the betaproteobacterial family *Methylophilaceae*, suggesting an interaction between methanotrophic and methylotrophic microorganisms that allowed for rapid methane oxidation. From our microcosms, we estimated methane oxidation rates of up to 871 nmol of methane per gram sediment and day. This implies that more than 50% of methane at the seep is removed by microbial oxidation at the sediment-water interface, based on previously reported *in situ* methane fluxes. The organic carbon produced was further assimilated by different heterotrophic microbes, demonstrating that the methane-oxidizing community supported a complex trophic network. Our results provide valuable eco-physiological insights into this specialized microbial community performing an ecosystem function of global relevance.

Introduction

Methane is the most abundant hydrocarbon in the atmosphere, and acts as a harmful greenhouse gas (Reeburgh, 2007). Approximately one third of the global methane flux to the atmosphere is derived from natural sources (Judd et al., 2002b). Reports on the contribution of oceanic methane emissions, primarily originating from natural cold seeps along continental margins (Etiope, 2012), vary from 1 to 10% of the total flux (Kvenvolden et al., 2001; Judd et al., 2002b). The methane flux from the subsurface sea bed, however, is even higher (Reeburgh, 2007). Biological activity of methane-oxidizing microorganisms in seafloor sediments and the water column considerably reduces the amount of methane that reaches the atmosphere. These microorganisms, termed methanotrophs, use methane as their sole carbon and energy source. The methanotrophs act as a 'benthic filter' (Boetius and Wenzhöfer, 2013) modulating methane emission from the sea, and supply methane-derived carbon to a broad range of other organisms. Hence, in the seep environment, methanotrophs carry out a key role in the microbial community that is comparable to autotrophic primary producers, and their activity is affected by the microbial satellite community present (Yu and Chistoserdova, 2017). To understand the modulation of methane emission by the benthic filter, various studies have targeted microbial communities at methane seep areas, especially in the deep sea (see (Boetius and Wenzhöfer, 2013) for a review). Deep-sea sediments are typically characterized by fine-grain particles that limit the circulation of pore water. As the deep-sea seafloor is not influenced by hydrodynamic forces from waves or tidal movement, stable layers with steep hydrogeochemical gradients exist. Oxygen is consumed within the first few millimeters of the sediment through the degradation of organic matter deposited by sedimentation of particulate organic carbon (de Beer et al., 2006; Glud, 2008). Aerobic methane oxidation is hence restricted to a thin layer of sediment, or occurs in microbial mats covering the sediment (Boetius and Wenzhöfer, 2013; Ruff et al., 2016; Paul et al., 2017). In subsurface layers, anaerobic oxidation of methane (AOM) by methanotrophic archaea in combination with sulfate-reducing bacteria takes place, typically

representing the predominant process for methane removal beneath the seafloor (Knittel and Boetius, 2009; Boetius and Wenzhöfer, 2013).

Shallow methane seeps, in contrast, can feature highly permeable sandy sediments, which allow advection-driven pore water circulation that introduces oxygen into deeper layers. The gas flow upwards additionally leads to a downstream of oxic sea water (O'Hara et al., 1995). Further, hydrodynamic forces result in mixing of the sediment and impede the formation of overlying microbial mats. Hence, in contrast to the stable conditions in deep-sea sediments, shallow sediments comprise a highly variable and heterogeneous environment with fluctuating oxygen concentrations. The frequent influx of oxygen restricts the highly oxygen-sensitive AOM consortia to deeper sediment layers (Knittel and Boetius, 2009). Thus, aerobic methane oxidation in the upper layers and at the sediment-water interface might be the predominant process for methane removal at shallow seeps.

Methane originating from depths below 100 m typically does not reach the sea surface due to dissolution processes of methane bubbles and oxidation of dissolved methane (Schmale et al., 2005; McGinnis et al., 2006). Hence, deep-sea seeps play little to no role in atmospheric methane emission. For shallow methane seeps, models suggest site specific parameters such as depth and initial bubble size along with aqueous methane concentration and upwelling flows to be major factors determining methane emission (Leifer and Patro, 2002; McGinnis et al., 2006). Emission from such shallow seeps has been estimated as 310 g CH_4 m^{-2} $year^{-1}$ at the Kattegat coast, Denmark (Dando et al., 1994), up to 550 g CH_4 m^{-2} $year^{-1}$ at Torry Bay, UK (Judd et al., 2002a), 260 g CH_4 m^{-2} $year^{-1}$ at Isla Mocha, Chile (Jessen et al., 2011), and 400 g CH_4 m^{-2} $year^{-1}$ at the Santa Barbara Channel, CA, USA (Luyendyk et al., 2003). The total emissions of the small Kattegat and Torry Bay seeps, covering an area of only a few thousand square meters, are in the range of one metric ton per year, while the Isla Mocha and Santa Barbara Channel seep, covering several square kilometers, are estimated to release 800 to 7200 metric tons of methane per year into the atmosphere.

Little is known about the identity and filter function of aerobic methanotrophic bacteria in such shallow seep areas. In this study, we investigated the diversity and function of aerobic methanotrophs at a shallow methane seep located off the coast of the Island of Elba, Italy, at only 12 meters depth. Discovered in 1995, the Elba shallow methane seep is located in a tectonically-active site (Greve et al., 2014) and is characterized by a gentle, constant bubbling of gas, consisting of up to 73% (Meister et al., 2018) to more than 85% abiotic methane (Ruff et al., 2016; Sciarra et al., 2019), leading to an efflux of 145 g CH_4 m^{-2} $year^{-1}$ into the water column (Sciarra et al., 2019). A previous investigation of AOM at the seep site revealed predominantly sulfur-coupled methane oxidation by consortia resembling those found in deep-sea seeps, but restricted to sediment layers more than 20 cm below the seafloor (Ruff et al., 2016). AOM exhibited only a low methane removal efficiency, and the authors concluded that aerobic methane oxidation is probably more important at this site (Ruff et al., 2016).

Here, we explored the microbial community in the top 2-3 centimeters of the sediment at the Elba methane seep, and its potential for methane oxidation. The aims of our study were (I) to determine the activity of aerobic methanotrophs and estimate their efficiency in methane removal, (II) to identify the key players of methane oxidation active in the oxic sediments, and (III) to follow the flux of methane-derived carbon through the microbial community, assessing the role of methanotrophs as key suppliers of organic carbon at the seep. We combined a ^{13}C-methane stable isotope probing (SIP) approach with metagenomics, to obtain metagenome-assembled genomes (MAGs) of the microorganisms present, as well as metaproteomics, to verify their predicted metabolic functions and assess their activity. This allowed us to gain an understanding of structure and function of the specialized, methanotrophy-driven microbial community at the methane seep.

Results

Activity of methanotrophs in microcosms and estimation of the benthic filter efficiency

A rapid consumption of methane was observed in microcosms containing sediment and water from the Elba shallow methane seep, when supplemented with 1% (v:v, headspace) of ^{12}C- or ^{13}C-methane. Methane consumption started immediately after setup of the microcosms. After 7 days of incubation, methane consumption rates of 439 ± 42 nmol d^{-1} g $sediment^{-1}$ (average of microcosms with ^{12}C and ^{13}C methane, n = 12, ± SD) were observed, with no difference between ^{12}C and ^{13}C incubations (Figure 1). As the high consumption rates led to frequent depletion of methane, we increased the headspace concentration to 2% after 25 days of incubation. This resulted in a significant increase ($p < 0.001$, Student's *t*-test) of methane consumption to 871 ± 123 nmol d^{-1} g $sediment^{-1}$ (average of microcosms with ^{12}C and ^{13}C methane, n = 8, ± SD) (Figure 1). For individual microcosms, methane consumption up to 2.26 µmol d^{-1} g $sediment^{-1}$ was observed (Dataset S1). In comparison, reported methane consumption rates for AOM at the same site were only up to 200 nmol d^{-1} g $sediment^{-1}$ under 1.5 atmospheres of CH_4:CO_2 (90:10) (Ruff et al., 2016).

Using the average rate of methane consumption for 2% headspace concentration, we estimated the annual methane consumption in the Elba methane seep. Based on the sediment porosity given in (Ruff et al., 2016), we calculated a methane consumption of approximately 12 mol m^{-2} $year^{-1}$ (Supplementary Information). Previous studies have reported a gas flow of 0.72 L m^{-2} d^{-1} from the sediment (Sciarra et al., 2019), containing approximately 85% (v:v) methane, resulting in a release of 9 mol m^{-2} $year^{-1}$ methane into the water column. Hence, based on our estimated rates, more than 50% of the methane flowing through the sediment is consumed at the sediment water interface. Indeed, this is likely a considerable underestimation of the *in situ* methane consumption. The methane concentration in the water phase of our microcosms was approximately 22 µM (2% methane), according to calculations based on Henry's Law (Supplementary Information). *In situ* concentrations at the Elba methane seep are up to one order of magnitude higher, with 50 µM to

550 µM reported for pore water (Ruff et al., 2016). Considering the increase of methane consumption observed in our microcosms when increasing the headspace methane concentration from 1% to 2%, *in situ* consumption could be considerably higher than our estimates. Given that this aerobic removal of methane at the sediment-water-interface exceeds previously reported AOM rates (Ruff et al., 2016), we aimed to explore the function of the underlying microbial methane oxidizing processes.

Identifying the key methane oxidizers

We used an integrated approach combining different 'omics' techniques with SIP to elucidate the key players responsible for the methane consumption observed in our microcosms. Taxonomic profiles of the microbial communities in the microcosms sampled after 25, 45 and 65 days were investigated by metaproteomics to determine the dominant microbial taxa. The majority of peptides identified were consistently related to *Proteobacteria*, with *Alphaproteobacteria* and *Gammaproteobacteria* (including *Betaproteobacteriales*, based on the current Silva taxonomy release 132 (Quast et al., 2013)) being the dominant classes (Figure S1). At the family level, the presence of various taxa implicated in C_1 metabolism was revealed, including *Methylococcaceae* (*Gammaproteobacteria*), *Methylophilaceae* (*Betaproteobacteriales*) and *Rhodobacteraceae* (*Alphaproteobacteria*) (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Ruff et al., 2015). To identify the active methanotrophs, ^{13}C incorporation in peptides extracted from the microcosms amended with ^{13}C-methane was investigated. Peptides related to *Methylococcaceae* as well as *Methylophilaceae* showed ^{13}C relative isotope abundances (RIA) and incorporation patterns suggesting a direct uptake of ^{13}C from methane (Figure 2, Figure S2). Peptides of *Rhodobacteraceae*, however, as well as those of several other taxa, showed incorporation patterns that suggested ^{13}C uptake by cross-feeding rather than by direct uptake of a ^{13}C-labelled substrate. The ^{13}C isotopologue patterns acquired using SIP-metaproteomics allow a differentiation between such modes of carbon assimilation (Seifert et al., 2012; Taubert et al., 2012).

195 Furthermore, PCR analysis targeting key functional genes for C_1 metabolism was linked with DNA-SIP
196 by investigating the heavy DNA fractions obtained from ^{13}C microcosms. The presence of *pmoA*,
197 encoding the small subunit of the copper-dependent particulate methane monooxygenase (pMMO),
198 as well as of *xoxF*, encoding a lanthanide-dependent methanol dehydrogenase (MDH) (Keltjens et al.,
199 2014; Taubert et al., 2015; Howat et al., 2018) were observed. However, no *mmoX* encoding the
200 alpha-subunit of soluble methane monooxygenase (sMMO), or *mxaF*, encoding a calcium-dependent
201 MDH were found. Interestingly, *pmoA* sequences were exclusively affiliated with *Methylococcaceae*,
202 while *xoxF* sequences were mainly affiliated with *Methylococcaceae*, *Betaproteobacteriales* and
203 *Rhodobacteraceae* (Figure S3). Complementary functional analysis of the metaproteomes likewise
204 revealed that peptides of the pMMO, covering all three subunits PmoCAB, were exclusively affiliated
205 to *Methylococcaceae*. No peptides of other methane oxidizing enzymes, such as sMMO or methyl-
206 coenzyme M reductase (Friedrich, 2005), were found. Peptides of methanol dehydrogenases were
207 exclusively related to XoxF and not to MxaF, and were affiliated to multiple taxonomic groups,
208 including *Methylococcaceae*, *Methylophilaceae* and different *Alphaproteobacteria* (Figure 3). Hence,
209 while multiple taxa were potentially involved in downstream functions like the oxidation of methanol
210 to formaldehyde, only *Methylococcaceae* were able to catalyze the first step in methane
211 degradation, the oxidation of methane to methanol.

212 To explore the key players for methane oxidation more closely, we conducted SIP-metagenomics by
213 Illumina MiSeq sequencing of the DNA obtained from heavy fractions of the ^{13}C microcosms. Ten
214 million MiSeq reads were assembled and binned, resulting in 99 metagenome-assembled genomes
215 (MAGs), with two MAGs considered complete genome drafts (> 90% completeness, < 5%
216 contamination (Parks et al., 2015; Vollmers et al., 2017a)) and another eight intermediate quality
217 genome drafts (> 70% completeness, < 10% contamination (Bishara et al., 2018) (Figure S4).
218 Surprisingly, eighteen different MAGs affiliated with *Methylococcaceae* were found (Table 1),
219 indicating multiple closely related methane oxidizers. To provide a more accurate taxonomic
220 classification and to estimate relatedness between the different *Methylococcaceae* MAGs, we

performed phylogenetic analysis based on amino acid sequences of single copy marker genes (SCMG) (Wu et al., 2013). All *Methylococcaceae* MAGs contained marker genes that were most closely related to those of *Methylomonas* spp., creating a sister lineage of this genus (Figure 4A). The amino acid identity between the MAGs was typically less than 85%, indicating that indeed multiple closely related species were present.

Genes encoding subunits of pMMO, i.e., *pmoC*, *pmoA* and *pmoB,* were present exclusively in MAGs affiliated with *Methylococcaceae*. The same MAGs typically also contained genes of an ortholog to the *pmoCAB* operon, dubbed *pxmABC* (Figure S5). These orthologs also encode copper-dependent monooxygenases, which are potentially involved in methane oxidation under oxygen limited and nitrite rich conditions (Kits et al., 2015b; Kits et al., 2015a). Potentially linked to these putative alternative pMMOs, several MAGs contained genes involved in denitrification, such as *narG* and *napABC*, encoding nitrate reductases, and *nirS*, encoding nitrite reductase. The expression of the *pmoCAB* genes was confirmed for multiple MAGs (Table 2, Table S1), but no expression of *pxmABC* genes, as well as of the genes involved in denitrification, was observed. No other functional genes for methane-oxidizing enzymes were observed in the metagenomes. Based on both genomic and proteomic data, these bacteria utilized XoxF-type MDHs for oxidation of methanol to formaldehyde. The classification of the MDH genes was verified by phylogenetic analysis using a custom reference database of *xoxF* and *mxaF* genes, clearly placing the detected genes in the *xoxF5* clade (Figure S6). Furthermore, genes of the tetrahydromethanopterin (H_4MPT) pathway for formaldehyde oxidation, as well as key genes of the ribulose monophosphate (RuMP) cycle for formaldehyde assimilation, 3-hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase, were expressed. The identified key players hence showed the typical metabolic traits of type I methanotrophs, in agreement with their taxonomic affiliation within the *Gammaproteobacteria* (Trotsenko and Murrell, 2008).

The gene expression profiles of the different *Methylococcaceae*, as well as the enrichment of their DNA in the heavy fraction and the ^{13}C incorporation in their peptides, demonstrated that several of

these closely related bacteria were active and responsible for methane oxidation in the microcosms. Considering the heterogeneity of the sediment present at the methane seep, these bacteria can have differing environmental preferences, and so their distribution might be driven by hydrogeochemical factors beyond the availability of methane. Hence, despite their taxonomic similarity, these bacteria might inhabit different environmental niches.

Role of non-methanotrophic methylotrophs

In addition to the key methanotrophs, non-methanotrophic organisms affiliated with *Methylophilaceae* were also found to be highly active in the microcosms, as deduced from ^{13}C incorporation. Despite their lack of the ability to oxidize methane, evident from metaproteomic, metagenomic and functional gene data, the ^{13}C incorporation patterns in their peptides were indistinguishable from those of the methanotrophic *Methylococcaceae* (Figure 2, Figure S2), resembling a direct uptake of a ^{13}C labelled substrate (Seifert et al., 2012). Phylogenetic analysis of the six MAGs related to *Methylophilaceae* in our metagenomic dataset, based on amino acid sequences of SCMGs, demonstrated an affiliation with *Methylophilus* spp. and *Methylotenera* spp. (Figure 4B). Functional classification of peptides identified in the metaproteomics analysis showed the presence of XoxF-type methanol dehydrogenases affiliated with the *Methylophilaceae* (clades XoxF4 and XoxF1, Figure S6), as well as enzymes of the H_4MPT pathway for formaldehyde oxidation and the RuMP cycle for formaldehyde assimilation, supporting a methylotrophic lifestyle. The identified peptides could be mapped to several of the six *Methylophilaceae* MAGs observed (Table 2, Table S1), indicating that also from this taxon, different methylotrophs were active in our microcosms.

As no genes or proteins involved in methane oxidation in the *Methylophilaceae* in our microcosms were present, we can exclude that these organisms used methane directly as a carbon source, and instead have more likely been labelled by cross-feeding. For cross-feeding organisms, a shift in the peptide RIA with incubation time can often be detected when newly synthesized, ^{13}C-labelled compounds from the primary consumers mix with pre-existing, unlabelled compounds (Seifert et al.,

2012; Taubert et al., 2012). In our study, we observed such shifts, for instance, in autotrophic *Nitrospirales* (Figure S2) that became labelled due to the enrichment of the carbonate pool in the incubations by $^{13}CO_2$ released from ^{13}C-methane oxidation. However, a low concentration of the respective pre-existing compound, e.g., caused by a starvation period or a rapid uptake by the cross-feeding organisms, will not result in sufficient amounts of intermediately labelled peptides to be detected by metaproteomics analysis. Given the presence of key methylotrophic functions in the *Methylophilaceae*, the most likely explanation for the ^{13}C labelling of these organisms is the uptake of ^{13}C methanol released from the methanotrophic *Methylococcaceae*, implying a transfer of carbon from methanotrophs to methylotrophs.

Interestingly, further putative methylotrophs related to the alphaproteobacterial family *Rhodobacteraceae* were present and active in our microcosms, but showed only indirect ^{13}C incorporation patterns slowly increasing in RIA over time (Figure 2). The low ^{13}C-labelling ratio observed indicated a much slower growth rate than for *Methylophilaceae*. Of 14 MAGs affiliated with *Alphaproteobacteria*, seven were related to the *Roseobacter* clade within the *Rhodobacteraceae*, while the remaining were related to *Hyphomonadaceae*, *Stappiaceae* and an unknown *Rhodobacterales* family (Table 1, Figure S7). Only for one of the MAGs affiliated with the *Roseobacter* clade was a gene encoding a *xoxF*5-type MDH found, as well as the corresponding gene product, indicating that the majority of these bacteria were not able to utilize methanol. Nevertheless, most of the 14 MAGs revealed a metabolic potential for C_1 utilization, typically including glutathione- and tetrahydrofolate-(THF)-dependent pathways for C_1 oxidation/reduction as well as key genes of the serine cycle for formaldehyde assimilation, including hydroxypyruvate reductase, glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA reductase. Furthermore, in two of the MAGs affiliated with the *Roseobacter* clade, a gene encoding ribulose bisphosphate carboxylase required for CO_2 fixation was present. The coverage of our metaproteomic analysis was insufficient to verify the metabolism of these alphaproteobacterial organisms. The potential for C_1 utilization suggested that they might assimilate other C_1 compounds potentially

299 derived from methane oxidation, such as formaldehyde. However, the ^{13}C RIA in the peptides
300 affiliated with *Alphaproteobacteria* was significantly lower than that of *Methylophilaceae* ($p < 0.001$
301 for all time points, Student's *t*-test), while not significantly different to the autotrophic *Nitrospirales*.
302 This suggested that some of these organisms could have assimilated carbon from CO_2, while using C_1
303 compounds as energy source (Figure 5). However, the lower RIA observed for *Alphaproteobacteria*
304 might also result from recycling of unlabelled organic compounds in the microcosms. Hence, while
305 our results strongly indicate that the different alphaproteobacterial taxa were continuously active
306 and oxidized C_1 compounds to gain energy in our microcosms, the nature of their carbon source
307 remains uncertain.

308 Discussion

309 Previous studies indicated that the activity of methane oxidizing microorganisms leads to a massive
310 reduction of methane emission from marine seeps. Boetius and Wenzhöfer summarized that
311 between 20 and 80% of methane released from cold seeps of continental slopes is removed by this
312 process, depending on the seep environment, with fluid flow rate and oxygen availability as
313 influential parameters (Boetius and Wenzhöfer, 2013). Here we confirmed that this notion holds true
314 for a shallow methane seep near Elba, characterized by highly permeable sandy sediment that allows
315 an increased oxygen circulation into deeper layers. The methane oxidation potential estimated at 12
316 mol m^{-2} $year^{-1}$, based on rate measurements in microcosms, was in the same range as the methane
317 flux in the water column of 9 mol m^{-2} $year^{-1}$, measured *in situ* (Sciarra et al., 2019), indicating that a
318 major portion of the methane is removed at the sediment-water interface before reaching the water
319 column (Figure 5).

320 We identified members of the *Methylococcaceae* within the order *Methylococcales* as the key
321 methane oxidizers. Previous studies indicated that *Methylococcales* are typically found at high
322 relative abundance at methane seeps, independent of seep hydrogeochemistry and geographic
323 location (Ruff et al., 2015). Here we showed that the key methane oxidizers present at the Elba seep

formed a sister lineage to *Methylomonas* sp. within the *Methylococcaceae*, potentially comprising a new genus, and that multiple closely related organisms of this taxon were present. This co-occurrence of bacteria from the same functional guild suggests the existence of different niches for methane oxidizers at the sediment-water interface. Parameters like the availability of oxygen and other electron acceptors, the methane concentration and the presence of alternative reduced molecules might drive the distribution of methane oxidizers with different metabolic capabilities. The presence of *pxmABC* genes hints to the potential for nitrite-dependent methanotrophy in the Elba sediments, given suitable conditions (Kits et al., 2015b; Kits et al., 2015a). Furthermore, the substrate-specificity of pMMO-like proteins is often not clear (Tavormina et al., 2013; Khadka et al., 2018), so some *Methylococcaceae* might additionally be capable of oxidizing alternative compounds like short chain alkanes. These divergent metabolic traits would allow the methanotrophs to occupy various niches and thrive under different biogeochemical conditions. Such a functional redundancy provides multiple advantages for ecosystem functions, such as enhanced stability against environmental disturbances (Griffiths and Philippot, 2013). In the shallow, sandy sediment, disturbances can easily occur, e.g., by hydrodynamic forces like waves and currents, or by seasonal changes (Ruff et al., 2016). Moreover, the adaptation of microorganisms to specific environmental niches optimizes their function and hence results in a fine-tuning of the methane oxidation machinery.

Furthermore, the association of methanotrophs with non-methanotrophic methylotrophs seems to be of major importance for the efficiency of methane oxidation. Our results suggested a transfer of methane-derived carbon from the *Methylococcaceae* to methylotrophs related to *Methylotenera* spp. and *Methylophilus* spp. of the *Methylophilaceae*. Interactions of *Methylococcaceae* with other bacteria, e.g., leading to aggregate formation, have been previously reported at deep-sea methane seeps (Ruff et al., 2013). Typically, *Methylophaga* spp. or other gamma- and alphaproteobacterial species are the most abundant methylotrophs associated with the methanotrophic *Methylococcaceae* (Lösekann et al., 2007; Ruff et al., 2013; Ruff et al., 2015; Paul et al., 2017).

Methylophilaceae related to *Methylotenera*/*Methylophilus* spp., in contrast, are only rarely observed at marine methane seeps (Ruff et al., 2013; Paul et al., 2017). With the notable exception of the OM43 clade (Giovannoni et al., 2008), members of the *Methylophilaceae* family are typically not abundant in marine environments, and seem to prefer environments with lower salinity such as estuaries or freshwater (Kalyuzhnaya et al., 2006; Kalyuzhnaya et al., 2012; Deng et al., 2018). Intriguingly, in sediments of Lake Washington (WA, USA), a well-studied freshwater lake featuring high methane fluxes, cooperations between *Methylococcaceae* and *Methylophilaceae* have been observed as well (Kalyuzhnaya et al., 2008; Beck et al., 2013). Incubation experiments revealed specific relationships between *Methylosarcina* spp. and *Methylophilus* spp. at high oxygen concentrations, as well as *Methylobacter* spp. and *Methylotenera* spp. at lower oxygen concentrations (Hernandez et al., 2015). Synthetic culture experiments with methanotrophic and non-methanotrophic isolates from Lake Washington also revealed *Methylomonas* spp. to be included in such partnerships, and to be highly competitive (Yu et al., 2017). While the non-methanotrophic partners of such interactions obviously benefit from the release of methanol from the methanotrophs, the gain for the methanotrophs is still unclear. An exchange of public goods, such as vitamin B12, or interspecies electron transfer contributing to methane activation have been discussed (Yu and Chistoserdova, 2017). Regardless, the interaction of methanotrophs and methylotrophs is a common theme across various environments featuring high methane fluxes, and seems to be a major factor for efficient functioning of the benthic methane filter (Ho et al., 2014).

Methanol and other C_1 compounds are typically produced in marine environments as byproducts of algal growth or decomposition of organic compounds such as osmolytes, resulting in concentrations in the nM to µM range (Naqvi et al., 2005; Beale et al., 2015). Hence, methylotrophs that degrade these compounds are commonly found in marine habitats. These methylotrophs, however, are distinctly different from those present at methane seeps, and are typically dominated by members of the *Roseobacter* clade, the *Methylophilaceae* group OM43 or the SAR11 clade (Giovannoni et al., 2008; Sun et al., 2011; Zhuang et al., 2018). In our microcosms, we found members of the

376 *Roseobacter* clade and other *Alphaproteobacteria* with the genetic potential for C_1 utilization. These
377 bacteria showed low, but consistent activity throughout 65 days of incubation. To succeed in the
378 open sea water, these bacteria are optimized for the uptake of the low concentrations of organic
379 compounds present, and usually utilize various C_1 compounds as well as multi-carbon substrates
380 (Brinkhoff et al., 2008), and typically exhibit slow growth rates. In our microcosms, we observed an
381 uptake of methane-derived carbon by these bacteria, but were unable to discern whether they
382 assimilated methanol or other C_1 compounds as byproducts of methane oxidation, or multi-carbon
383 compounds released by the primary C_1 utilizers, or if they fixed CO_2 and used organic carbon
384 compounds solely as energy sources. Such a chemoorganoautotrophic lifestyle, often supported by
385 anoxygenic photosynthesis, has been reported for various marine methylotrophs, termed
386 "methylovores" (Sun et al., 2011; Pinhassi et al., 2016). Hence, although the methane seep recruits a
387 distinct and specific community of C_1-utilizing organisms, apparently the typical marine
388 methylotrophs can also sustain their activity in this environment, and potentially benefit from the
389 increased levels of organic compounds produced by the methanotrophs.

390 Interestingly, all methanotrophs and methylotrophs of the *Methylococcaceae*, *Methylophilaceae* and
391 other *Alphaproteobacteria* detected in our incubations employed lanthanide-dependent, XoxF-type
392 methanol dehydrogenases instead of the calcium-dependent methanol dehydrogenase MxaFI. The
393 high diversity of *xoxF* gene sequences in marine habitats, especially *xoxF4* and *xoxF5*, as well as their
394 prevalence over *mxaF* gene sequences, has previously been described (Ramachandran and Walsh,
395 2015; Taubert et al., 2015). The lanthanides required for these enzymes, belonging to the rare earth
396 elements, are typically present in sufficient concentrations in coastal environments from sediments
397 or coastal run-off, despite their low solubility (Elderfield et al., 1990; Keltjens et al., 2014).

398 In summary, we showed that the microbial community present in the oxic sediments at the Elba
399 methane seep is highly efficient in methane removal, exceeding the methane oxidation rates
400 reported for AOM at this site (Ruff et al., 2016), likely due to the high oxygen levels in the sediment
401 precluding AOM. We identified members of the *Methylococcaceae* as the key players of aerobic

methane oxidation, and obtained several genome drafts of different active, closely related members of this group. We observed a tight association of these methanotrophs with non-methanotrophic methylotrophs of the *Methylophilaceae*, likely through exchange of methanol, contributing to the efficiency of methane oxidation. Finally, methane-derived carbon was also transferred to other microorganisms not able to utilize methanol, supporting the hypothesis that methanotrophs fuel a complex trophic network and can be considered as primary producers in the methane seep environment. The gain of knowledge on methane removal by the 'benthic filter' at shallow seeps provided by our study will facilitate future estimations of the global methane budget, and highlights the relevance of methanotrophs as model systems to study principles of microbial interactions.

Experimental Procedures

Sample collection and microcosm setup

Samples of oxic sediment from the top 2-3 cm and water were collected in May 2014 by divers from a shallow methane seep located off the coast of Elba, Italy (42° 44.628' N, 10° 07.094' E), in 12 m water depth. Five 50 ml BD Falcon™ tubes were filled with ~100 g of sediment each, and two 1 L bottles were filled with seawater from a maximum of 50 cm above the sediment surface. Samples were transported and stored at 4°C until the start of the SIP experiments at the University of East Anglia, United Kingdom, four days after sampling. Microcosms were set up in 120 ml serum bottles with 20 g of sediment and 25 ml of seawater each, and marine ammonium mineral salts (MAMS) were added to a final concentration of 1% of full-strength medium. Microcosms were spiked with 1% (v:v, headspace) ^{13}C-labelled or unlabelled (^{12}C) methane (six of each), and incubated at 25°C in a shaking incubator (50 rpm). Headspace methane concentrations were monitored using gas chromatography (Supplementary Information). When the headspace concentrations in all microcosms were below 0.1% (v:v), additional methane (1-2%, v:v) was added. Duplicate ^{12}C and ^{13}C microcosms were sacrificed for DNA and protein extraction after 25, 45 and 65 days of incubation.

DNA and protein extraction and DNA-SIP

Combined DNA and protein extractions were performed from microcosms as well as from untreated sediment (T0) according to a previously described protocol (Taubert et al., 2012) with minor modifications (Supplementary Information). Extracted DNA was subjected to fractionation using CsCl gradients, and fractions containing ^{13}C-labelled DNA were selected as previously described (Neufeld et al., 2007; Grob et al., 2015) with minor modifications (Supplementary Information).

Amplicon and metagenomic sequencing

PCR amplicons for 454 sequencing were obtained from selected fractions using the following primer sets and conditions: The *pmoA* gene encoding the β-subunit of particulate methane monooxygenase was amplified by nested PCR using primer pairs A189F/A682R (Holmes et al., 1995) and A189F/mb661R (Costello and Lidstrom, 1999) as previously described (Horz et al., 2005). The *mmoX* gene encoding soluble methane monooxygenase subunit A was amplified by nested PCR using primer pairs mmoX166f/mmoX1401r (Auman et al., 2000) and mmoX206f/mmoX886r (Hutchens et al., 2004) as described. The *xoxF4*, *xoxF5* and *mxaF* genes encoding different methanol dehydrogenases were amplified using primer pairs xoxF4f/r, xoxF5f/r (Taubert et al., 2015) and mxaF1003f/mxaF1555r (McDonald and Murrell, 1997) using PCR conditions as described by these authors. Combined and purified triplicate PCR products were subjected to 454 pyrosequencing (GS FLX Titanium system, MR DNA, Shallowater, TX, USA). Sequencing data were processed using mothur (v.1.35.1) (Schloss et al., 2009) for quality control, demultiplexing, and removal of barcodes and primers as previously described for other functional genes (Taubert et al., 2015). Sequences were binned to OTUs with a 97% identity threshold and chimeras were removed using USEARCH (v7.0.1090) (Edgar, 2013). Phylogeny was assigned using Megan (v.5.1.5) (Huson et al., 2011) and a previously described pipeline for functional genes (Dumont et al., 2014). Raw data are available at the National Center for Biotechnology Information (NCBI) database under bioproject PRJNA524087.

For metagenomic sequencing, separate libraries were prepared from total DNA from untreated sediment (T0) as well as from ^{13}C-labelled DNA obtained from the duplicate microcosms of each of the three time points. Metagenomic DNA was sheared using a Covaris S220 sonication device (Covaris Inc., MA, USA) with the following settings: 55 s 175 W, 5% Duty factor, 200 cycles of burst, 55.5 µl. Library preparation was done using the NEBNext® DNA Library Prep kit for Illumina® (E6040, New England BioLabs® Inc., Ipswich, MA, USA). Sufficient material for sequencing (15 - 20 µg) was obtained from SIP fractions without further amplification. Metagenome sequencing was then performed on an Illumina MiSeq machine using v3 chemistry (600 cycles).

Metagenome reads were adapter clipped and quality trimmed using Trimmomatic v0.32 (Bolger et al., 2014). Low complexity reads were removed using the DUST approach of prinseq-lite v0.20.4 (Schmieder and Edwards, 2011) with a cutoff of 15, and residual phiX-contaminants were filtered out using FastQ Screen (Wingett and Andrews, 2018). Overlapping read pairs were then merged using FLASH 1.2.11 (Magoč and Salzberg, 2011).

For each time point and for the untreated samples, an individual metagenome assembly was produced by coassembling the corresponding libraries from experimental replicates using megahit v1.0.5 (Li et al., 2015). Read coverage of assembled contigs was determined by mapping using Bowtie2 (Langmead and Salzberg, 2012). Each metagenome was then binned using Maxbin v.2.1.1 (Wu et al., 2016). Bins were subsequently decontaminated using a z-score based differential coverage approach previously described (Vollmers et al., 2017b; Pratscher et al., 2018). Bins with a high likelihood of originating from the same species were identified based on similarity of coverage profiles across all time points and subsamples, as well as by the presence of nearly identical universal marker genes. Any such related bins were merged and coassembled by extracting the respective reads from all corresponding time points and reassembly using megahit. Completeness and potential contamination of the final binned MAGs was estimated using CheckM (Parks et al., 2015). Phylogenetic trees to elucidate taxonomic relationships for metagenome-assembled genomes based on concatenated amino acid alignments of taxon-specific single copy marker genes were constructed

using the ezTree pipeline (Wu, 2018). The shotgun metagenome reads, corresponding assemblies, as well as binned MAGs with estimated completeness > 70% and contamination < 10% are available at the NCBI database under bioproject PRJNA522277.

SIP-metaproteomics

Sample preparation for metaproteomics analysis was done as previously described (Grob et al., 2015). Mass spectrometry was performed on an Orbitrap Fusion MS (Thermo Fisher Scientific, Waltham, MA, USA) (Supplementary Information).

Proteome Discoverer (v1.4.0288, Thermo Scientific) was used for protein identification and the acquired MS/MS spectra were searched against the NCBI nr database with taxonomy set to Archaea and Bacteria using the Mascot algorithm, and against protein sequences derived from all acquired MAGs using the SequestHT algorithm. Trypsin was chosen as cleavage enzyme, allowing a maximum of two missed cleavages. The precursor mass tolerance (MS) was set to 10 ppm, the fragment mass tolerance (MS/MS) was 0.05 Da. Carbamidomethylation of cysteine was considered as fixed and oxidation of methionine was set as dynamic modification. Peptide spectrum matches (PSMs) were validated using Percolator (v2.04) with a false discover rate (FDR) < 1% and quality filtered for XCorr ≥ 2.25 (for charge state +2) and ≥ 2.5 (for charge state +3). Identified proteins were grouped by applying the strict parsimony principle (Nesvizhskii and Aebersold, 2005). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD013378.

Taxonomic classification of peptides was done by the lowest common ancestor method using UniPept (Mesuere et al., 2018). Identification of ^{13}C-labelled peptides and quantification of ^{13}C incorporation was done by comparing measured and expected isotopologue patterns, chromatographic retention times and fragmentation patterns as previously described (Seifert et al., 2012; Taubert et al., 2012). For each taxonomic group of interest, ^{13}C incorporation was quantified in 10 peptides per time point, 5 from each replicate microcosm.

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The authors declare no conflict of interest.

Supplementary information is available at ISME Journal's website.

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Figure and Table Legends

Figure 1: Methane consumption in microcosms with sediment from the Elba methane seep. Values given are the cumulative amount of methane consumed in the microcosms. Separate averaged values for microcosms with ^{12}C-methane and microcosms with ^{13}C-methane are depicted by cross and diamond symbols , respectively. Error bars indicate standard deviation. Arrows indicate time points of methane addition. Brackets display the amount of methane (% headspace, v:v) of each addition and number of replicate microcosms (n) each supplemented with ^{12}C- or ^{13}C- methane.

Figure 2: ^{13}C incorporation into peptides of different bacterial taxonomic groups. Values depict (A) the ^{13}C relative isotope abundance (RIA), i.e., the amount of carbon replaced by ^{13}C, and (B) the labelling ratio, i.e., the abundance of ^{13}C-labelled compared to unlabelled molecules, of peptides specific to the given taxonomic groups after incubation of sediment for 25, 45 and 65 days with ^{13}C-methane. Values are based on n = 10 peptides per time point, error bars show standard deviation.

Figure 3: Functional classification of identified peptides. The numbers of peptides affiliated to different enzymes and pathways of different functional categories relevant for C_1 metabolism are shown. Colors depict the taxonomic distribution of the peptides in each functional category based on the lowest common ancestor of each peptide. Peptide identification is based on metaproteomics analysis of samples from microcosms with ^{12}C-methane of all three time points (n = 6). The peptides were identified using NCBI nr and the metagenome-assembled genomes obtained in this study as reference databases. MMO: methane monooxygenase, MDH: methanol dehydrogenase, FAE: formaldehyde-activating enzyme, H4MPT: tetrahydromethanopterin pathway for formaldehyde oxidation, THF: tetrahydrofolate pathway for formaldehyde oxidation, glutathione: glutathione pathway for formaldehyde oxidation, formate DH: formate dehydrogenase, RuMP: ribulose monophosphate pathway, based on the key enzymes 3-hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase. For the serine cycle, the key enzymes hydroxypyruvate reductase,

753 glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA
754 reductase were taken into account. For the Calvin cycle, the key enzyme ribulose-1,5-bisphosphate
755 carboxylase/oxygenase was taken into account.

756 **Figure 4: Phylogenetic affiliation of the key methanotrophs and methylotrophs identified at the**
757 **Elba methane seep.** (A) Phylogenetic tree representing key methanotrophs, based on a concatenated
758 amino acid alignment of 36 single copy marker genes with a total of 6 329 positions. Only
759 metagenome-assembled genomes (MAGs) related to *Methylococcaceae* with at least 50%
760 completeness are shown. *Pseudomonas oryzae* (*Pseudomonadales*) was included as an outgroup to
761 root the tree. (B) Phylogenetic tree representing key methylotrophs, based on a concatenated amino
762 acid alignment of 94 single copy marker genes with a total of 21 475 positions. Only MAGs related to
763 *Methylophilaceae* with at least 35% completeness are shown. *Sulfuricella denitrificans*
764 (*Gallionellaceae*) was included as an outgroup to root the tree. Both trees were inferred with the
765 Approximately-Maximum-Likelihood approach of FastTree using the JTT-CAT model for amino acid
766 evolution, local support values were calculated using the Shimodaira-Hasegawa test from 1 000
767 resamples. The scale bars indicate the number of amino acid changes per site.

768 **Figure 5: Conceptual overview of communal methane metabolism at the Elba seep.** The character C
769 in red indicates methane-derived carbon. OC: organic carbon compounds released from the primary
770 methane utilizing community of *Methylococcaceae* and *Methylophilaceae*. *Methane consumption
771 of the microbial community estimated based on average consumption rates in microcosms from this
772 study. [+]Methane flux from sediments to hydrosphere as reported in Ruff et al., 2015 (Ruff et al.,
773 2015).

774 **Table 1: Statistics for metagenome-assembled genomes affiliated with *Methylococcaceae*,**
775 ***Methylophilaceae* and other *Alphaproteobacteria*.** Taxonomic relationships were elucidated based
776 on concatenated amino acid alignments of taxon-specific single copy marker genes using the ezTree

pipeline (Wu, 2018). [1]Based on CheckM analysis (Parks et al., 2015). N50: 50% of the genome assembly is contained in scaffolds equal to or larger than this value.

Table 2: Presence and expression of functional genes for C_1 metabolism in metagenome-assembled genomes. White fields indicate presence of functional genes for the respective function, red fields indicate expression of the encoded enzymes based on metaproteomics analysis. Numbers in the fields indicate number of genes expressed / number of genes present. [1]Based on key genes 3-hexulose-6-phosphate synthase and 3-hexulose-6-phosphate isomerase. [2]Based on key genes hydroxypyruvate reductase, glycerate 2-kinase, malate thiokinase, malyl coenzyme A lyase, isocitrate lyase and crotonyl-CoA reductase. [3]Based on key gene ribulose-1,5-bisphosphate carboxylase/oxygenase.